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Original Article

The effect of advanced paternal age on the outcomes of assisted reproductive techniques among patients with azoospermia using cryopreserved testicular spermatozoa

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Abstract

Objective: To determine whether advanced male age influences the outcome of intracytoplasmic sperm injection (ICSI) following the cryopreservation of spermatozoa obtained through testicular sperm extraction (TESE).

Materials and Methods: Data were collected from infertile couples suffering from azoospermia who underwent TESE and ICSI from January 1998 to August 2010. There were 212 ICSI cycles using extracted testicular sperm after cryopreservation in this retrospective clinical analysis. The participating men all underwent testicular biopsy and subsequent tissue cryopreservation in a single academic tertiary care medical center. Those cryopreserved sperm were used during consecutive intracytoplasmic sperm injection treatment cycles. Female partners underwent individualized controlled ovarian hyperstimulation programs.

Results: A total of 184 ICSI cycles were divided into the following two evaluation designs: (1) total cycles irrespective maternal age; (2) ICSI cycles with maternal age <34 years. Male partners were stratified into age categories at 5-year intervals (31–35 years, 36–40 years, and 41–51 years) in these two designs. In the first design, most outcomes of assisted reproductive techniques were similar during the three groups, but the maternal age is much lower in the first group, and the mean number of retried oocytes and estradiol level on the day of human chorionic gonadotropin injection was significantly higher in the first group. In the second design, the outcome of intracytoplasmic sperm injection and clinical factors including the estradiol level on the day of human chorionic gonadotropin injection, the number of retrieved oocytes, the rate of cleaved oocytes, the number of transferred embryos, the numbers of transferred good embryos, the clinical pregnancy rate per transfer cycle and the implantation rate were similar among the three groups of women aged <34 years after adjusting for female age.

Conclusions: There is insufficient evidence to demonstrate an unfavorable effect of advanced paternal age on the fertility outcome for TESE-ICSI. The thawed testicular spermatozoa from males aged ≤40 years did not have an adverse impact on ICSI outcomes.

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Keywords: azoospermia; cryopreservation; paternal age; testicular sperm extraction

Introduction

The outcome of assisted reproductive technology (ART) procedures is known to be influenced by multiple factors, including the etiology of infertility, patient age, the type of ovarian stimulation, and the level of follicular phase estradiol (E2). The subsequent number and quality of oocytes and the number of embryos transferred are affected by the different regimens of ovarian stimulation [1]. The impact of these factors on the outcome of intracytoplasmic sperm injection (ICSI) following testicular sperm extraction (TESE) has also been examined, including a comparison of fresh and freeze-thawed testicular sperm as well as the length of

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cryopreservation [2–4]. The negative impacts of advancing female age on pregnancy outcomes are well known [5–7]. Female fertility decreases after age 35 years and declines sharply after age 39 years [8,9], and advanced female age has been associated with poor ART results [10–13].

Compared with maternal age, few studies have focused on the effect of paternal age on ART outcomes, and some of the results are conflicting [9]. The review by Kidd et al [14] evaluating the effects of male age on fertility and semen quality concluded that advanced male partner age is associated with lower semen volume, sperm motility, and morphology, but no change in sperm concentration was noted. This review found that advanced male age negatively affected fertility, particularly in men aged >50 years. Recently, some studies have also evaluated the effect of paternal age on semen characteristics and have found similar declines in total sperm count, total motile sperm count, and total semen volume [9,15,16].

According to a review from 2011 [9], there was no significant relationship between advanced paternal age and ICSI outcomes among ejaculated sperm, but there were some conflicting results, such as the decreased blastocyst formation in men >50 years [13]. However, the effects of male age on the spermatozoa obtained from cryopreservation of TESE tissue remain unknown. The aim of this study was to investigate the impact of advanced male age on outcomes following cryopreservation and TESE.

Materials and methods

Study population

This was a 14-year study that began in 1997. All participating patients underwent a complete physical examination of their genitalia to evaluate the anatomy of the seminal vesicles, prostate, distal vasa deferentia, and ejaculatory ducts by an experienced urologist in our hospital. Male patients diagnosed as having azoospermia underwent TESE with routine cryopreservation at our hospital. Thawed testicular spermatozoa were subsequently used for a total of 212 ICSI cycles until August 2010. After excluding men <31 years, 184 ICSI cycles were included in our analysis.

Testicular tissue extraction, preparation of testicular sperm, and cryopreservation of testicular spermatozoa

Patients exhibiting azoospermia agreed to undergo TESE subsequent to their routine work-up. A formal scrotal exploration was performed prior to the surgical testicular-tissue extraction procedure. The cryopreservation of testicular spermatozoa was as described previously [3,17]. Briefly, the effluent that contained the sperm was put in culture medium (Medicult, Copenhagen, Denmark) and centrifuged for 20 minutes at 300 × g, after which it was washed twice. Test yolk buffer containing glycerol was added to the sperm suspension, and 500-μL aliquots of the mix were transferred to sterile polypropylene tubes. Then, 10 μL of the suspension was delaminated to examine the presence of fully formed spermatozoa by phase-contrast microscopy. The tube was put in a 37 °C incubator for 15 minutes and shifted to a 4 °C refrigerator for another 15 minutes. This was followed by incubation at 20 °C for another 15 minutes. During the last step, the tubes were left at −60 °C for 5 minutes. Finally, the tubes were stored in liquid nitrogen.

Ovarian stimulation

Ovarian stimulation and oocyte retrieval were performed as described in previous studies [3,17]. Briefly, all patients underwent a long stimulation protocol using a gonadotropin-releasing hormone (GnRH) agonist (Luproid acetate; Takeda, Tokyo, Japan) therapy followed by the administration of a urinary follicle-stimulating hormone (FSH, Metrodin; Serono Laboratories Inc., Randolph, MA, USA) or administration of recombinant follicle-stimulating hormone (r-FSH; Gona-F Serono Laboratories Inc., Randolph, MA, USA) and transvaginal oocyte retrieval. Human chorionic gonadotropin (hCG; Pregnyl, N.V. Organon/Oss, The Netherlands) was administered (10,000 IU) intramuscularly when ultrasound revealed at least one follicle featuring a mean diameter of ≥17 mm and/or when serum estradiol levels exceeded 400 pg/mL. Oocytes were retrieved by transvaginal aspiration under ultrasound guidance 35–37 hours subsequent to the injection of hCG.

Following oocyte retrieval, the oocytes were exposed to type VIII hyaluronidase (80 IU/mL; Sigma Chemical Co., St Louis, MO, USA) for a period of 5–6 seconds. The oocytes were then separated from the surrounding cumulus cells by aspiration through a series of pipettes with decreasing inner diameters (commencing at 220 μm, and progressing through 200 μm, 180 μm, and 160 μm), in 100-μL droplets of human tubal fluid (HTF) medium.

ICSI procedure

The ICSI procedures were followed by the previous studies [3]. Briefly, a two-layer gradient was set for purifying the thawed specimens. Most of the spermatozoa that were selected under these two criteria had at least occasional tail twitching and normal morphology. The following modification was adopted for the preparation of sperm droplets: 1 μL of prepared sperm suspension was added to 2–4 μL of M2 medium (Medicult, Copenhagen, Demark) containing no polyvinylpyrrolidone.

Fertilization was confirmed 16–18 hours after insemination and was defined by the presence of two distinct pronuclei. If only one pronucleus was observed, a second evaluation was performed after 4 hours. Embryonic division and morphology were evaluated every 24 hours and classified according to the morphological grading system. Daily intravaginal luteal phase supplementation of 800 mg of micronized progesterone was started on the day of oocyte retrieval, and 5000 IU HCG was administered on Day 6 after oocyte recovery in all patients. Clinical pregnancy was defined as the identification of a gestational sac at 7 weeks of gestation by transvaginal sonography. Micronized progesterone was administered to prospective

mothers for a period of 4 weeks in cases of conception. We defined the implantation rate as the number of gestational sacs divided by the total number of transferred embryos.

Outcome measures

The primary outcome measure of this study was the live birth rate. Secondary outcome measures included the clinical pregnancy rate, the implantation rate, the fertilization rate, embryo development, and the mean number of transferred good embryos. A good embryo was defined as a Grade 1 or Grade 2 embryo. Embryos were graded by the number and symmetry of distinct blastomeres and by the percentage of total embryo volume consisting of cellular fragments on the day of embryo transfer.

Statistical analysis

Results are expressed as the mean ± standard error of the mean for numeric variables. Categorical variables are expressed as proportions (%). The statistical evaluation was performed using one way ANOVA, Fisher's test, and a χ^2 test. A p value <0.05 was considered to be statistically significant.

Results

A total of 212 ICSI treatment cycles were performed during the study period. After excluding men <31 years, male partners were stratified into age categories at 5-year intervals (31–35 years, 36–40 years, and >40–51 years). In Table 1, the mean age of the females in the first group was less than the other two groups due to the lack of age adjustment. This group also experienced less ovarian stimulation and had a greater number of oocytes and a higher estradiol level. The general embryo quality was lower in the first group, but the better quality of the transferred embryo was noted after selection. The clinical pregnancy rate, implantation rate, live birth rate, and abortion rate were similar across all three groups. The distribution of oligozoospermic and nonoligozoospermic azoospermia patients was similar in Table 1. In order to exclude the influence of maternal age to clearly elucidate the effect of paternal age, we excluded couples with a maternal age >34 years to control for this factor. In Table 2, female age was similar after matching for age. The distribution of oligozoospermic and nonoligozoospermic azoospermia patients was also similar. The dose of ovarian stimulation was also not significantly different. Similar numbers of retrieved oocytes were also noted, which indicated a similar ovarian reserve among the three groups. There was no statistically significant difference in the fertilization rates of the retrieved oocyte between groups. Additionally, the embryo quality was similar among groups, suggesting that the sperm and oocytes used in the procedure were of similar quality. The percentage of top-quality embryos used in these groups was similar. The implantation rate was not significantly different among the three groups. Additionally, the live birth rate did not decrease among those with advanced paternal age. These results are related to the good control of female factors.

Table 1
Influence of male age on intracytoplasmic sperm injection (ICSI) outcome with thawed testicular sperm in all women.

	Male age interval (y)		
	31–35	>35–40	>40
No. of ICSI cycle azoospermia type (nonobstructive:obstructive azoospermia)	84 (51/33)	56 (33/23)	44 (23/21)
Female age (y)	31.0 ± 0.4	33.4 ± 0.6[a]	34.2 ± 0.9[b]
Female factor with endometriosis or ovulation	3 (3.5)	6 (10)	2 (4.5)
Gonadotropin dose (Amp/75 IU)	30.9 ± 1.2	33.6 ± 2.2	33.7 ± 2.0
Mean no. of retrieved oocytes	8.4 ± 0.5	6.1 ± 0.5[a]	5.8 ± 0.4[b]
Total no. of injected oocytes	612	286	198
Total no. of fertilized oocytes	545	252	182
Mean rate of fertilization	88.3 ± 1.9	84.4 ± 3.7	92.2 ± 1.9
Total no. of cleaved oocytes	528	245	175
Mean rate of cleavage	96.9 ± 1.7	87.6 ± 4.1[a]	92.4 ± 3.5
No. of blastomeres on Day-2 embryos			
≤ 3	139 (26.3)	59 (24.1)	43 (24.6)
≥ 4	389 (73.7)	186 (75.9)	132 (75.4)
Embryo quality on Day-2 embryos			
Good	415 (78.6)	189 (77.1)[a]	141 (80.6)[a]
Poor	113 (21.4)	56 (22.9)	34 (19.4)
Mean no. of good embryo per transfer	2.9 ± 0.2	2.5 ± 0.3	2.2 ± 0.2
Estradiol level on hCG day	2220.3 ± 160.5	1646.9 ± 172.6[a]	1279.6 ± 118.2[a]
Clinical pregnancy rate per transfer	38/84 (45.2)	23/56 (41.1)	17/44 (38.6)
Implantation rate	54/251 (21.5)	21/135 (15.6)	27/110 (24.6)
Live birth rate	35/84 (41.7)	19/56 (33.9)	14/44 (31.8)
Abortion rate	7/84 (8.3)	4/56 (7.1)	3/44 (6.8)

Data are presented as mean ± standard error of the mean or n (%).

[a] The results are significantly different ($p < 0.05$) compared with the result in the age 31–35 years group; [b] The result are significantly different ($p < 0.05$) compared with the result in the age >35–40 years group.

Table 2
Influence of male age on intracytoplasmic sperm injection (ICSI) outcome with thawed testicular sperm in the women (aged ≤34 years).

	Male age interval (y)		
	31–35	>35–40	>40
No. of ICSI cycles azoospermia type (nonobstructive:obstructive azoospermia)	69 (45/24)	30 (20/10)	15 (10/5)
Female age (y)	30.4 ± 0.3	30.2 ± 0.6	29.8 ± 1.0
Female factor with endometriosis or ovulation	2 (2.8)	6 (20)[a]	0 (0)
Gonadotropin dose (Amp/75 IU)	31.4 ± 1.3	31.9 ± 2.9	27.3 ± 2.3
Mean no. of retrieved oocytes	8.5 ± 0.6	6.9 ± 0.7	7.3 ± 0.8
Total no. of injected oocytes	519	170	94
Mean no. of injected oocytes	7.4 ± 0.5	5.7 ± 0.7	5.7 ± 0.7
Total no. of fertilized oocytes	461 (88.8)	150 (88.2)	88 (90.5)
Mean rate of fertilization	88.6 ± 2.1	81.3 ± 2.9	93.1 ± 3.6
Total no. of cleaved oocytes	444 (96.3)	148 (98.7)	85 (94.7)
Mean rate. of cleavage	97.6 ± 1.5	86.0 ± 1.2[a]	97.8 ± 1.5
No. of blastomeres on Day-2 embryos			
≤3	109 (24.6)	28 (18.9)	21 (27.8)
≥4	335 (75.4)	120 (81.1)	64 (72.2)
Embryo quality on Day-2 embryos			
Good	344 (77.5)	113 (78.4)	64 (74.1)
Poor	100 (22.5)	35 (21.6)	21 (25.9)
Mean no. of good embryos per transfer	2.8 ± 0.2	2.4 ± 0.3	2.9 ± 0.3
Estradiol level on hCG day	2255.8 ± 183.7	1521.3 ± 182.7	1539.9 ± 223.9
Clinical pregnancy rate per transfer	32/69 (46.4)	13/30 (43.3)	7/15 (46.7)
Implantation rate	49/215 (22.8)	18/75 (24.0)	12/46 (34.4)
Live birth rate	23/69 (33.3)	11/30 (36.7)	6/15 (40)
Abortion rate	7/69 (10.1)	3/30 (10)	1/15 (6)

Data are presented as mean ± standard error of the mean or n (%).
[a] The results are significantly different ($p < 0.05$) compared with the result in the age 31–35 years group.

Discussion

Among most previous studies, the main impact of male aging on natural pregnancy outcome was decreased sperm parameters [14,18]. It is unknown whether IVF outcomes are affected by sperm from aging males. One paper mentioned the poor conception rate of IVF with a paternal age ≥40 years [19]. A recent review by Dain et al [9] suggests a significant decrease in blastocyst embryo formation associated with increased paternal age. Decreased semen volume and motility with increasing age were also mentioned in a cohort study, but they did not find an association between paternal age and the likelihood of live birth [7]. Based on those reviews, [7,9], there is no obvious evidence demonstrating a negative effect of advanced paternal age on IVF outcomes.

Although the sperm are selected via microscopy for ICSI procedures, it is unknown whether ICSI outcomes are affected by sperm from aging males. The influence of advanced male age on ICSI outcomes suffers from insufficient and conflicting data and not yet well established [16,19]. In our TESE group, the fertility rate in the aging groups (>40 years) was adequate. However, as mentioned above, some studies have suggested a negative trend in fertility with advanced male age [14,19]. A similar trend was noted when we separated patients into subgroups of paternal age (31–35 years, 36–40 years, 40–45 years, and >45 years). We found very large declines in the clinical pregnancy rate, implantation rate, and live birth rate with a paternal age >45 years. However, due to the limited number of cases, additional patients are required prior to when a conclusion regarding the influence of advanced male age (>40 years) can be drawn.

As many studies have suggested, advanced male age is related to the risk of chromosomal abnormalities in spermatozoa and might increase the spontaneous abortion rate [20,21]. However, the abortion rate in our study was similar between groups. Additionally, clinical data were similar and satisfactory between the groups in our study. Therefore, the excellent ART data in our study lead us to ask how the TESE-ICSI procedures can compensate for the negative effects of advanced male age. According to our review, several studies have suggested that DNA fragmentation is increased by the freeze–thaw procedure [22–25]. Additionally, our team demonstrated that the post-thaw vitality was significantly different from the vitality of fresh sperm [26]. According to those findings, we have two hypotheses. First, an age-dependent impact may play an important role in epididymal sperm maturation by disturbing sperm mitochondrial function [18,27]. We suggest that TESE is a good way to avoid the influence of poor environment of the seminiferous tubules (in the epididymis and the ejaculatory duct) that ejaculated sperm have to pass through in older males.

Second, we hypothesize that our cryo–thaw TESE procedures result in excellent selection in poor quality spermatozoa related with advanced paternal age. We suggested that TESE sperm with chromosomal abnormalities are affected more during the cryo–thaw TESE procedure. So the poor quality spermatozoa related with advanced paternal age are less likely to survive following the procedure. Therefore, we hypothesize that

the negative impact of paternal age related to the spermatozoa quality was bypassed after the selection system.

There are still some limitations that need to be acknowledged and addressed regarding the present study. First, the topic of this study can be only analyzed retrospectively. The impact of more advanced age (>40 years) needs to be established with a larger sample. Although many studies have suggested that advanced male age has negative impacts on the outcomes of natural pregnancy and artificial pregnancy following ART, it appears that these effects can be overcome by the use of the cryo–thaw TESE and ICSI procedures. Thawed testicular spermatozoa from men aged ≥40 years did not have an adverse impact on ICSI outcomes.

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